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חוק הפטנטים, תשכ"ז - 1967
PATENT LAW, 5727 - 1967

בקשה לפטנט
Application for Patent

לשימוש הלשכה
For Office Use

מספר: 121750 Number

תאריך: 11-09-1997 Date

הוקדם/נדחה
Ante/Post-dated

אני, (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)
(Name and address of applicant, and in case of body corporate-place of incorporation)

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(באנגלית) (English) Method for large scale mutagenesis and gene knockout in crop plants

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מבקשת פטנט from Application	*לבקשה/לפטנט to Patent/Appl.	מספר/סימן Number/Mark	תאריך Date	מדינת האגוד Convention Country
מס' No.....	מס' No.....			
מיום dated.....	מיום dated.....			
* יפוי כח: כללי / מיוחד - רצוף בזה / עוד יוגש P.O.A.: general / individual-attached / to be filed later -				
הוגש בעניין filed in..... case: 110696				
המען למסירת מסמכים בישראל Address for Service in Israel: Paulina Ben-Ami, Yeda Research & Development Co. Ltd., P.O.Box 95, Rehovot 76100				
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METHOD FOR LARGE SCALE MUTAGENESIS AND GENE KNOCKOUT IN CROP PLANTS

שיטה למוטאגנזה בקנה מידה רחב ולפגימה מכוונת של גנים בצמחי גידול

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ידע חברה למחקר ופתוח בע"מ
יישום חברה לפיתוח המחקר של
האוניברסיטה העברית בירושלים

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FIELD OF THE INVENTION

The present invention is in the field of plant genetics and relates to improved methods for gene mutagenesis, tagging and knockout in crop plants, particularly in tomato.

BACKGROUND OF THE INVENTION

The genome of higher plants contains about 50,000 genes, however a funtion has been ascribed for only few hundred plant genes.The isolation of new genes, and the knockout of sequenced genes whose function is not known, are essential for future improvement of crop plants by biotechnology approaches,

The *Arabidopsis* model system has contributed much to the remarkable advances in plant molecular biology during the last decade. The major reasons for the *Arabidopsis* success are its small size, short life cycle, small genome (Leutwiler et al., 1984) and easy transformability (Bechtold et al., 1993). These features facilitate the genetic dissection of any trait through screening of large populations saturated in mutants for the various genes involved in the trait. Populations mutagenized by ethyl methanesulfonate (EMS), fast neutron, T-DNA insertions, and transposon tags proved invaluable to plant biologists (Koncz et al., 1992). Nevertheless, despite the considerable advantages of *Arabidopsis*, it is not a crop, and the knowledge acquired in this species cannot always be applied to other plant species. Having a silique type of fruit makes *Arabidopsis* a good model for species of the Brassicaceae but not for those with a fleshy berry fruit.

Tomato (*Lycopersicon esculentum*) is one of the most important crops in the fresh vegetable market as well as in the food processing industry (Hille et al., 1989; Rick and Yoder, 1988). It is well known genetically; it has a relatively small diploid genome (n=12, C=1pg) with hundreds of mapped traits and molecular markers (Tanksley, 1993) and it is transformable (McCormick et al., 1986). Moreover, it offers a good model for other crop

species whose fruit is also a fleshy berry. However, a major obstacle to further advance tomato genetics is the lack of populations required for large scale screening of mutants. Such mutagenized populations could contain at least one mutant allele for every tomato gene, thus making it possible to achieve saturated mutagenesis in tomato. DNA damaging agents such as EMS,(Hildering and Verkerk, 1965; Schoenmakers et al., 1991; Wisman et al., 1991) X-rays (Hildering and Verkerk, 1965)or fast-neutrons (Verkerk, 1971) have been used in tomato, although to a relatively limited extent compared to similar efforts in *Arabidopsis*. A few hundred mutant tomato lines, available through the Tomato Genetic Resource Center, have been described, but no stocks of mutagenized M2 seeds, originating from a large population of M1 plants, are available for screening mutations in new genes.

Insertional mutagenesis by T-DNA tagging is not practical in tomato as transformation procedures are still laborious. Transposon tagging on the other hand proved to be a promising approach for mutagenesis and gene tagging in tomato. The *Ac/Ds* transposable element family was shown to be active in tomato (Yoder et al., 1988) and patterns of *Ac/Ds* transposition in this species were described (Carroll et al., 1995; Osborne et al., 1991; Rommens et al., 1992; Yoder et al., 1988) Tomato lines were produced containing *Ds* elements that were mapped in the tomato genome (Knapp et al., 1994; Thomas et al., 1994) These lines make it possible to take advantage of the preferential insertion of *Ac/Ds* at nearby sites (Dooner and Belachew, 1989; Jones et al., 1990) The *Ac/Ds* tagging system was used to tag and isolate several genes, such as *Cf9*, a locus responsible for *Cladosporium* resistance (Jones et al., 1994); *Dwarf*, a gene encoding a cytochrome p450 homolog (Bishop et al., 1996); and *DCL* which controls chloropast development (Keddie et al., 1996).

An efficient way to determine function of sequenced genes, and thus identify "useful" genes is by targeted knockout. A gene knockout system has been developed in maize (the maize "gene machine"): it is based on identifying a mutation in a target gene through screening of a large population composed of 48,000 plants, each of which contains different mutations caused by transposable element insertions. Currently, in tomato, the

most important crop in the fresh vegetable market and in the food processing industry, it is not possible to knockout specific genes.

Based on the results of Cooley et al. (1996), Ac/Ds transposons can also be used for site-selected insertion into target genes in tomato. Using this technique, insertions into a target gene are detected by PCR screening of DNA from pooled plants carrying independent transposition events, using one primer in the transposon and one in the gene to be knocked out. The results of Cooley et al (1996) however are based on insertions in somatic plant tissues and are not practical for gene knockout. Developing "a tomato gene machine" similar to that of maize would require considerable efforts and field facilities as tens of thousands of plants must be grown to maturity to detect the mutant of interest.

DEFINITIONS

Transposon - a natural DNA sequence able to move or jump to different locations in the genome. Through insertion into existing genes and consequent gene disruption, the transposon causes mutation of said genes. Transposons were found in bacteria, Drosophila, yeast, mematodes, plants and mammals.

Transposable element - transposon.

Transposase - protein expressed by an autonomous transposon which binds to the terminal regions of a transposon, and mediates transposon excision and transposition to another location in the genome.

Autonomous Transposon - an element that codes for a transposase and has terminal regions recognized by the transposase for its catalytic activity and thus transposes autonomously. Mutations caused by autonomous transposons are unstable. Examples of autonomous transposons are the Ac (Activator) transposons of maize.

Non-Autonomous Transposon - an element that contains the terminal regions recognized by a transposase but does not code therefor, and thus needs a transposase supplied in trans in order to excise and transpose to another location in the genome. Examples of non-autonomous transposons are the Ds (Dissociation) transposons of maize, that can be used together with an autonomous, e.g. Ac, transposon.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide an improved method for large-scale mutagenesis, gene tagging and knockout in crop plants by utilizing a miniaturized crop plant as a system (host).

Thus, in one aspect, the present invention relates to a method for large-scale mutagenesis and gene knockout in crop plants, which method comprises:

(a) providing a miniature cultivar of a crop plant, said miniature cultivar having the following characteristics: (i) it is of reduced size in comparison to to the commercial cultivar of the same species; (ii) it can be grown at a plant density of at least 10-fold higher than standard growth conditions used for a commercial cultivar of the same species and can still yield reproductive organs under these conditions; and (iii) it is able to cross with a commercial cultivar of the same species;

(b) treating said miniature cultivar with a mutation-inducing agent or generating mutations of said miniature cultivar by insertion of a mobile DNA sequence; and

(c) identifying mutants or knockout of specific genes in said miniature cultivar by screening of large mutant-containing plant populations.

The method of the present invention is suitable for crop plants used as food, for ornamental purposes and for the wood industry such as, but not being limited to, tomato, maize, fruit trees, potato, flowers.

The miniature cultivar of the crop plant may be developed from natural or induced mutations, by genetic engineering or by treatment of the crop of interest with plant growth factors. The size of the crop being reduced signficantly, this facilitates to grow plants at a density of at least 10-fold higher than in standard field conditions, and thus to analyze large plant populations on small areas. In the case of the tomato miniature cultivar Micro-Tom, as described in the examples hereinafter, the plants are grown at a density of about 200-fold higher than in standard field conditions. New mutants or knockouts obtained in the miniature cultivar can be transferred to a commercial background by standard crosses with the crop and by segregating out the miniaturizing gene(s) or transgene(s).

Any mutagenesis technique can be applied according to the invention including, but not being limited to, chemical treatment, irradiation, T-DNA or with transposons from the host plant or from an heterologous origin. Gene knockout can be achieved by detection of site selected insertion of transposons or by other methods which require the screening of large plants population.

Chemical treatment for production of mutants of the miniature cultivar can be carried out by known techniques with mutagens such as ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), methyl-N-nitrosourea (MNU), bleomycins and the like. Mutation can also be done by known techniques through irradiation with UV, γ-irradiation, X-rays and fast neutrons.

The mobile DNA sequence may be a T-DNA or a transposon .T-DNA insertion may be carried out by known methods via Agrobacterium (Hoekema et al., 1983; US Patent No. 5,149,645) and transposon insertion may be done by known methods described before (Fedoroff et al., 1984; US Patents No. 4,732,856 and No. 5,013,658).The transposable element used may be an autonomous transposon, a non-autonomous transposon or an autonomous/non-autonomous transposon system, e.g. the maize Ac/Ds transposon system.

The screening of mutant plants is carried out in large populations, namely thousands, of plants. Identification of mutants can be done visually or by assaying for specific traits such as, but not being limited to, response to hormones, to minerals, to pathogens, to herbicides, and the like, by known techniques used in plant biology.

Identification of knockout in a specific gene can be done, for example, by methods involving PCR screening using a first primer corresponding to a sequence of the transposon or of the T-DNA and a second primer corresponding to a sequence of the gene to be knocked out (the target gene).

The improved method of the invention will facilitate identification of genes of commercial value, e.g. genes affecting fruit ripening, genes to improve yield and/or quality of the plant; to isolate new genes, e.g. genes related to sugar content in the fruit, to minerals uptake, etc; to introduce new genes in classical plant breeding programs, and to

isolate tissue specific promoters using a gene trapping methodology built-in within the transposon.

In another aspect, the present invention relates to a method for generating a library of mutants in crop plants comprising:

(a) introducing T-DNA or a transposable element into a miniature cultivar of a crop plant, said miniature cultivar having the following characteristics: (i) it is of reduced size in comparison to to the commercial cultivar of the same species; (ii) it can be grown at a plant density of at least 10-fold higher than standard growth conditions used for a commercial cultivar of the same species and can still yield reproductive organs under these conditions; and (iii) it is able to cross with a commercial cultivar of the same species;

(b) generating a large population of mutant plants of the miniature cultivar wherein each individual plant of said population contain a different T-DNA or transposon insertion;

(c) extracting DNA from pools of the mutant plants of step (b);

(d) identifying a pool of mutant plants containing an insertion in the gene to be knocked out (the target gene) by screening the DNA of the T-DNA- or transposon-carrying plants by PCR using a first primer corresponding to a sequence of the T-DNA or of the transposon and a second primer corresponding to a sequence of the target gene, and amplifying the PCR product only in the positive pools;

(e) identifying individual mutant plants from within the positive pool using DNA extracted from individual plants from the positive pool by PCR using a first primer corresponding to a sequence of the T-DNA or of the transposon and a second primer corresponding to a sequence of the target gene.

In a particular embodiment, the method of the invention is applied to tomato and he miniature cultivar of tomato used is the miniature-dwarf-determinate *Lycopersicon esculentum* cultivar, Micro-Tom.

The invention thus provides a method for large-scale mutagenesis and gene knockout in tomato comprising the following steps:

(b) treating miniature-dwarf-determinate *Lycopersicon esculentum* cultivar, Micro-Tom.with a mutation-inducing agent or generating mutations of said miniature cultivar by insertion of a mobile DNA sequence; and

(d) identifying mutants or knockout of specific genes in said miniature tomato cultivar by screening of large mutant-containing tomato plant populations.

In another aspect, the invention provides a method for generating a mutant tomato gene library comprising:

(a) introducing T-DNA or a transposable element into a miniature-dwarf-determinate *Lycopersicon esculentum* cultivar, Micro-Tom;

(b) generating a population of mutant plants of the Micro-Tom wherein each individual plant of said population contains a different T-DNA or transposon insertion;

(c) extracting DNA from pools of the mutant plants of step (b);

(d) identifying a pool of mutant plants containing an insertion in the gene to be knocked out (the target gene) by screening the DNA of the T-DNA- or transposon-carrying plants by PCR using a first primer corresponding to a sequence of the T-DNA or of the transposon and a second primer corresponding to a sequence of the target gene, and amplifying the PCR product only in the positive pools; and

(e) identifying individual mutant plants from within the positive pool using DNA extracted from individual plants from the positive pool by PCR using a first primer corresponding to a sequence of the T-DNA or of the transposon and a second primer corresponding to a sequence of the target gene.

According to this aspect of the invention, the transposable element may be selected from an autonomous transposon, a non-autonomous transposon and an autonomous/ non-autonomous transposon system, and is preferably an autonomous/ non-autonomous transposon system, e.g. the maize Ac/Ds transposon system.

The major bottleneck to produce a tomato "gene knockout machine" is the considerable time and effort required to produce and handle very large plant populations. The present invention enables large scale production of transposon-mutagenized plants, faster and with much less effort than with current production techniques. It is estimated that 100,000

different transposon-mutagenized plants are necessary to produce a tomato gene machine. The production of such a library is a feasible goal with the present invention. It enables to knockout almost any desired gene by identifying a line which carries a transposon into the target gene. The knockout identification is done by screening pools of transposon-carrying plants with PCR, using one primer in the target gene and one primer in the transposon.

According to the present invention, a tomato model system was developed which can greatly enhance the study of tomato genetics and the ability to isolate important genes. This system is based on the miniature-dwarf-determinate *Lycopersicon esculentum* cultivar, Micro-Tom (Micro tomato), originally bred for home gardening purposes (Scott and Harbaugh, 1989). This cultivar can be grown at high density, up to 1357 plants per square meter; it has a short life cycle, yielding mature fruits within 70-90 days from sowing; and it can be transformed at frequencies of up to 80% through Agrobacterium-mediated transformation of cotyledons. Moreover, it differs from the standard tomato cultivars by only two major genes. Therefore, any mutation or transgene can be conveniently studied in Micro-Tom's background, and when needed, transferred into a standard background.

According to the present invention, the growth conditions and transformation for this cultivar were optimized, and the screening of 20,000 EMS mutagenized M2 plants derived from 9,000 M1 individuals were carried out. Mutants with altered pigmentation or modified shape of leaves, flowers and of fruits were found. An *Ac/Ds* transposable element enhancer trap system (Fedoroff and Smith, 1993) and a gene trap system (Sundaresan et al., 1995) were introduced into Micro-Tom and found active. Thus the Micro-Tom cultivar opens the prospect to reach saturated mutagenesis in tomato, to tag or knockout any gene and become a model system for tomato, as well as for fruit development in all species with a fleshy fruit.

DESCRIPTION OF THE DRAWINGS

Fig. 1 shows Micro-Tom response to growth conditions. The studied growth traits are indicated within each box with the range of values (minimum-maximum) given in parenthesis. Each trait, given as percentage of the maximum value for this trait, is expressed as a function of the root volume (bottom scale), or of the plant density (upper scale). The following traits were measured: Days to anthesis, the average number of days from sowing to anthesis; Days to ripening, the average number of days from sowing to fruit color turning; Plant height, the height (in cm) from the soil surface to the first inflorescence; No. leaves, the number of leaves on the main stem; Plant yield, the total fruit weight (in g) per plant; No. fruit, the number of fruits per plant; Fruit weight, the average weight (in g) of a fruit; and No. seeds, the average number of seeds per plant. Error bars were too small to be drawn.

Fig. 2 shows Micro-Tom wild type and mutant phenotypes. (A) Micro-Tom plants grown in nursery trays with root compartment of 13 ml (top left), 33 ml (top right) 90 ml (bottom left) and 200 ml (bottom right). (B) A wild type Micro-Tom mature plant, grown in a 90 ml compartment, is shown with a scale bar. The plant is 5-6 cm tall (not including roots) and fruits have a diameter of 1.5 to 2 cm. (C) Micro-Tom plants grown at a density of 226 plants/m^2 in a nursery. (D-H) EMS-generated M2 plants with a mutant phenotype. Plants carrying fruits with a persimon (D), pear (E) or oblong (F) fruit shape are shown. Several M2 plants with a chlorophyll mutant phenotype were found (yellow leaves) (G). An M3 family derived from a single M2 plant was found to segregate at a 3:1 ratio for anthocyanin (purple) pigmentation in the leaves (H).

Fig. 3 shows schematic representation of constructs transformed into Micro-Tom. Constructs DsG and DsE (Sundaresan et al., 1995) were used for gene trapping and enhancer trapping, respectively. Constructs Bam35S-Ac and Ds378-GUS were used for enhancer trapping (Fedoroff and Smith, 1993). Sequences similar to *Ac* are shown in gray, with the terminal inverted repeats shown as gray arrows. Constructs are flanked by the right (RB) and left (LB) borders of their respective T-DNA. The B-Glucuronidase gene (GUS) is fused to *Ac* weak promoter in Ds378-GUS, to the minimal -1 to -46

promoter region (black box) of the 35S in DsE; or to an Arabidopsis intron followed by three acceptor splice sites (black box) in DsG (Sundaresan et al. 1995). Resistance to kanamycin ($Kana^r$) or hygromycin ($Hygro^r$) is conferred by the neomycin phosphotransferase or aminocyclitol phosphotransferase genes, respectively. Sensitivity to naphthalene acetamide (NAM^s) is conferred by the indole acetic hydrolase gene. Ds mobility is achieved by crossing the Ds-containing plants (DsG, DsE and Ds378-GUS) with a transposase-producing plant transformed with Bam35S-Ac. In this construct, *Ac* transposase is produced under the control of the 35S promoter fused to an *Ac* element whose 5' terminal region, up to the unique BamHI site has been deleted. Chlorosulfuron resistance ($Chlorosulfuron^r$) is obtained upon excision of the Ds element from the Ds378-GUS-containing construct and activation of a mutated acetolactate synthase gene from Arabidopsis (Fedoroff and Smith, 1993). Excision footprints (Ex1 and Ex2) were obtained upon excision of Ds378-GUS in F1 of crosses between Bam35S-Ac and Ds378-GUS and amplified with primers pr1 and pr2. The sequence flanking Ds378-GUS is shown above Ex1 and Ex2. The underlined sequence indicates the host duplication flanking *Ac* insertion site in the original *wx-m7* maize allele from which Ds378-GUS was derived.

Fig. 4 shows markers used for transposition selection. Three-week-old Micro-Tom plants following three sprays (one every day) with 300 mg/l kanamycin (A). Kanamycin resistant plants, transformed with Bam35S-Ac (A, top panel) are compared to wild type, sensitive plants of the same age (A, bottom panel). White leaves develop at the shoot tip in sensitive plants. Plants transformed with Ds378-GUS are resistant to 20 µg/ml hygromycin (B, left) while wild type Micro-Tom is sensitive (B, bottom right). Plants, transformed with Bam35S-Ac are sensitive to 0.25 µg/ml naphthalene acetamide (C, left) while the wild type is resistant (C, right). Wild type plants grown on 100 p.p.b. chlorosulfuron are sensitive (D, left); plants transformed with Ds378-GUS have low resistance (D, middle); and F2 plants (Bam35S-Ac X Ds378-GUS), in which a germinal *Ds* excision event occurred, are fully resistant (D, right). X-Gluc staining of F1 (*Ds* X transposase) plants shows blue sectors (E-F). The promoter-less GUS reporter gene, in

DsG, was activated as seen by the blue color in the root of a ten-day-old F1 seedling (E). Young fruits, two weeks after anthesis and 1 cm large in diameter, were stained for GUS activity (F). No GUS activity was obtained in negative control plants such as the wild type or the Bam35S-Ac parent (F, top). GUS was activated in some of the F1 fruits (F, bottom).

Fig. 5 shows Southern blot of chlorosulfuron and hygromycin resistant plants. Genomic DNA was extracted from a transgenic plant homozygous for the Ds378-GUS construct (lane a); a plant homozygous for the Bam35S-GUS construct (lane b); the F1 plant of the cross between these two plants (lane c); and the derived F2 plants which were resistant to 2 ppm chlorosulfuron and to hygromycin (lanes d-l). DNA was digested with *Hind*III and run on a 0.8% agarose gel, transferred to a nylon membrane and hybridized with an internal 1 kb GUS probe. The arrow points to the 8 kb band from the Bam35S-GUS *Ds* parent.

DETAILED DESCRIPTION OF THE INVENTION

The present invention shows the feasibility of utilizing a miniaturized crop plant as a model system for the study of a target crop and for its large-scale mutagenesis.

Applying this concept to tomato, it is shown herein that Micro-Tom can be used as a new tool for the study of tomato genetics. This cultivar sets fruit even when grown at high density (up to 1357 plants/m^2), and its short life cycle facilitates screening up to four generations per year. These attributes make it an efficient system for screening large mutagenized plant populations and open the prospect to reach saturated mutagenesis in tomato. In addition, Micro-Tom can be transformed efficiently, thus accelerating characterization of transgenes in tomato: it takes about 100 days from the innoculation of cotyledons to the harvest of transgenic fruits. Therefore, mutants or transgenes can be produced and studied in Micro-Tom and, if needed, tested in standard tomato cultivar background. Because the two major genes responsible for the Micro-Tom type are recessive, dominant traits can be analyzed in a standard background already in the F1

generation. One more generation is required to transfer recessive genes to a standard background.

In one embodiment of the present invention, we have focused on the utilization of Micro-Tom for mutagenesis. The limited infrastructure for mutagenesis in tomato, compared to *Arabidopsis*, impedes progress in genetic analysis in this species. As shown herein, Micro-Tom can alleviate this problem: a 14,000 M2 plant population derived from 9,000 M1 plants was grown on 100 m^2. Moreover, it took only one person's work and a six month period (M1 was grown in spring and M2 in summer 1996) to produce this population. Even though the EMS mutagenesis described here was relatively mild, as suggested by the fact that less than 1% albino plants were found, a large number of mutants was recovered in this experiment. Since today only a few hundred tomato mutants have been described, it is likely that many new mutant genes are present in our M2 population.

All the M2 families that were derived from individual M1 plants and showed a mutant phenotype segregated in a 3:1 (dominant: recessive) ratio. This suggests that in Micro-Tom, under the experimental conditions used here, gametes are derived from a single cell present in the embryo of the mature seed at the time of mutagenesis. These data are in agreement with previous reports (Hildering and Verkerk, 1965; Verkerk, 1971) suggesting that between one to three cells give rise to the sporocyte in mutated tomato plants.

Although transposon tagging systems have been previously described in tomato (Carroll et al., 1995; Knapp et al., 1994; Rommens et al., 1992; Yoder et al., 1988), there is no previous report in the literature of an enhancer and gene trapping system in tomato. Similarly, according to the present invention, we introduced into tomato two systems for selection of unliked transposition: one system based on NAM sensitivity and kanamycin resistance (Sundaresan et al., 1995), and a second system based on the excision-insertion selection (Fedoroff and Smith, 1993), which takes advantage of the efficient detection of kanamycin-sensitive plants in tomato (kanamycin being in the T-DNA of the *Ds* donor site) and of hygromycin resistance contained within *Ds*. In addition, using the resistance to chlorosulfuron as an excision marker, coupled with other agronomical features of

Micro-Tom, enables to screen large tagged populations for enhancers and promoters as well as for gene isolation. Further, the recently described approach for site-selected insertions in somatic tissues of tomato (Cooley et al., 1996) can also be applied in Micro-Tom for stable germinal transposition events. In this respect, the *Ac/Ds* system shown to be active in Micro-Tom can also contribute to reverse genetics via gene knockout.

Thus Micro-Tom was developed according to the present invention into a model system for genetic studies in tomato. It complements the already available infrastructure for genetic studies in tomato by accelerating the characterization of transgenic plants, and facilitating the isolation of mutants, promoters and genes. Moreover, Micro-Tom can be used as a general model system for other commercially important crops (e.g. citrus, grapes, etc.) whose fruit, like tomato, is a berry. Any fruit gene, promoter, and mutant found in Micro-Tom can facilitate the study of genetics, physiology and metabolism of other botanically similar fruits.

The following examples are provided for illustration and are not to be construed as a limitation upon the claims.

Experimental Procedures

(a) Plant growth. Micro-Tom plants were sown and grown up to fruit maturity in nursery trays or pots. For the plant density experiment, each treatment corresponded to growth in a different root volume. For that purpose, plants were grown in a commercial nursery compartmentalized trays of 13, 33, 90 or 200 ml, or in pots of 465 ml capacity. There were two replications in each treatment, each consisting of 84 (13 ml treatment), 72 (33 ml), 63 (90 ml), 50 (200 ml) and 15 (465 ml) plants that were analyzed for each trait. For the EMS experiment, plants were grown in an insect-proof nethouse at the Weizmann Institute. Transgenic plants were grown in greenhouses.

(b) EMS mutagenesis. EMS-mutagenesis was performed on 15,000 Micro-Tom seeds. Seeds were imbibed from the morning (8:00 a.m.) to the afternoon (5:00 p.m.) on wet Whatman paper in Petri dishes, and then transferred to an Erlenmeyer bottle containing 150 ml of an unbuffered 0.7% EMS (Sigma) solution and incubated overnight (17:00 to 9:00 a.m.) at room temperature (22°C) with gentle shaking. Mutagenized seeds were

extensively washed, fan dried and sown on the same day (April 1996) in speedling trays. Compared to the control group, mutagenized seedlings were retarded in their growth and the percent of germination was reduced by ca. 25%. Approximately 10% of the M1 plants were sterile. M2 seeds were harvested from 9000 M1 plants. From 70 M1 plants, M2 seeds were harvested individually from each plant and 10-20 M2 plants were grown for each M1 plant in progeny rows. The rest of the M2 seeds were harvested in bulk, pooling one fruit from each M1 plant. Approximately 20,000 M2 seeds from the bulk harvest were sown in August 1996 giving rise to 14,000 fruit-producing M2 plants which matured towards the end of October 1996. M3 seeds were harvested in bulk.

(c) Micro-Tom transformation. Constructs Ds378-GUS and Bam35S-Ac, designed for transposon tagging and enhancer trapping (Fedoroff and Smith, 1993), were kindly provided by Nina Fedoroff. Constructs DsG and DsE which were designed for gene trapping and enhancer trapping, respectively (Sundaresan et al., 1995), were kindly provided by Venkatesan Sundaresan. Micro-Tom was transformed with constructs Ds378-GUS, Bam35S-Ac, DsE, and DsG using the following protocol. to optimize the conditions for Micro-Tom. Plates containing KCMS medium (Fillati et al., 1987) supplemented with 0.2 µg/ml 2,4-D and a tobacco feeder cells layer (Horsch et al., 1985) were incubated at 25°C under low light conditions for 24 hrs. Cotyledons of seven-day-old seedlings were cut near the petiole and at the tip, laid on a plate, and preincubated for 24 hrs at 25°C under low light conditions. The concentration of *Agrobacterium* strain LBA 4404 used for co-cultivation ranged from $5.\ 10^{-7}$ to $9.\ 10^{-7}$ cfu/ml, corresponding to an OD ranging from 0.4 to 0.5. Co-cultivation was carried out under the same conditions as preincubation and lasted for 48 hrs. Subsequently, the cotyledons were transferred to 2Z medium (Fillati et al., 1987) containing 100 µg/ml kanamycin and 400 µg/ml carbenicillin for 3-4 weeks, and then transferred again to 1Z medium with 200 µg/ml carbenicillin for 2-3 weeks. Shoots were then excised from the cotyledons and transferred to a rooting medium (MSSV) (Fillati et al., 1987) supplemented with 2 µg/ml IBA, 50 µg/ml kanamycin, and 100 µg/ml carbenicillin. Plantlets with roots appeared after 1-3 weeks and were then transferred to the greenhouse.

(d) Selection markers and GUS reporter. In addition to the kanamycin selection needed for transformation and the GUS reporter utilized in the trapping systems, a number of markers were used to select for transposition events (Fedoroff and Smith, 1993; Sundaresan et al., 1995). To that end, sterilized seeds were germinated and grown in 0.8% agar-containing Nitsh medium supplemented with one, or a with combination of the following compounds: 20 μg/ml hygromycin (Calbiochem); 0.25 μg/ml naphthalene acetamide (NAM, Sigma); and 100 p.p.b. or 2 p.p.m. chlorosulfuron (Dupont). GUS staining was done according to Jefferson (1987) and tissue clearing was done according to Beeckman and Engler (1994).

(e) DNA analysis. DNA was extracted from young leaves by the Dellaporta method (Dellaporta et al., 1983), with an additional phenol chlorophorm extraction. PCR reactions were performed using Promega *Taq* polymerase according to the manufacturer's recommendation, with 2.5 mM MgCl2 and 200 μM dNTPs in an MJ thermocycler. The following program was used: 2' denaturation at 94°C and 30 cycles of 1' at 94°C, 45" at 55°C, 1' at 72°C, and a final step of 5' at 72°C. The primers used to amplify *Ds* excision products were: pr2, 5' GGATAGTGGGATTGTGCGTC 3', which is complementary to sequences in the 35S promoter, and pr1, 5' GGATGATTTGTTGGGGTTTA 3' which is complementary to sequences in the ALS gene (Fig. 3). Bands of the expected size for excision products (ca. 322 bp) were extracted from the agarose gel, and DNA was purified using GenClean according to the manufacturer's instructions. These PCR products were cloned into a pGEM-T vector (Promega) and sequenced using the T7 or SP6 primers.

For Southern analysis, 2 μg genomic DNA was digested with *Hind*III, fractionated on a 0.8% agarose gels, and transferred to a nitrocellulose membrane purchased from MSI. Hybridization was performed according to manufacturer's instructions. An internal GUS fragment of 1 kb was amplified by PCR, radiolabeled by the random priming method (Feinberg and Vogelstein, 1983) and used as a probe for *Ds* detection.

Example 1. Growth habit and genetic makeup

Micro-Tom was grown from sowing to fruit maturation in nursery trays with root compartments of various sizes to determine the effect of density on plant growth, and on fruit and seed setting. Densities of 100 to 1357 plants per m^2, equivalent to root volumes ranging from 465 to 13 ml, were tested (Figure 1). Interestingly, some traits were barely affected by plant density. For example, the number of days from sowing to anthesis ranged from 37 to 40 days and the number of days from sowing to fruit ripening ranged from 75 to 82 days (Figure 1). When a control standard determinate tomato cultivar (cv. UC82) was grown under similar conditions, it failed to set fruit at the high densities (412-1357 plants/m^2), and at lower densities (100-226 plants/m^2), fruits developed only in some of the plants (data not shown). Some traits, such as plant yield, number of fruits or number of seeds per plant responded linearly to changes in plant density with a more than ten fold difference between the minimum and maximum values obtained in the experiment (Figure 1). The average fruit weight and plant height showed a lesser response to density with a two-fold difference between minimum and maximum values (Figure 1). Mature plants grown under the various density levels are shown in Figure 2A-B. Note that in Micro-Tom, all plant organs are reduced in a well-proportioned manner (with the exception of seeds which are near-normal in size). This contrasts with other tomato dwarf mutants which are compact in appearance and have large leaves compared to the overall plant size.

Micro-Tom was crossed with UC82, a determinate cultivar and with the indeterminate cultivarVF86. The F1 plants of both crosses were very similar in height to the "tall" parent indicating that the genes responsible for the Micro-Tom type are recessive. In the F2 population of the cross with UC82 there was a wide range of growth habit phenotypes. Six out of the 176 F2 plants analyzed were clearly of the Micro-Tom type, suggesting that it is controlled by two major recessive genes with the possible additional effect of modifiers. Based on the pedigree of Micro-Tom (Scott and Harbaugh, 1989), it can be hypothesized that *dwarf* and *miniature* are the two genes involved in the Micro-Tom phenotype. Test-crosses designed to check this hypothesis are in progress.

Example 2. EMS mutagenesis

In the M1 population (the treated generation), about 1% of the plants showed chlorophyll variegation. In M2 a total of 14,000 plants were grown in nursery trays and screened for mutant phenotypes (Figure 2 C). Out of this population, 111 chlorophyll mutants were found, including albinos, yellow (xanthophyll-like) and light green leaves (Figure 2G). Plants with a modified leaf shape, flower (petals) and fruit pigmentation were also found. Compared to the wild type round shape fruit, six plants showed an altered fruit shape in all their fruits, including phenotypes such as persimon shape (Figure 2D) and pear-shape (Figure 2E). Plants with oblong fruits had also long and narrow leaves (Figure 2F). Seventy M2 families derived from individual M1 plants were also screened for mutations. In five families we found a mutant phenotype that invariably segregated in a 3:1 ratio. One such family segregated for anthocyanin pigmentation in the leaves (Figure 2H).

Example 3 .Transposon tagging and enhancer trapping in Micro-Tom

The introduction of transposon tagging, and trapping systems in Micro-Tom can greatly facilitate the isolation of genes, promoters and enhancers in tomato. Constructs Ds378-GUS, Bam35S-Ac, DsE, and DsG (Figure 3) were transformed into Micro-Tom. These constructs contain the NPTII gene which confers resistance to kanamycin. NPTII can be used as a transformation marker to detect the presence of the T-DNA and to map *Ds* elements relative to their donor site in Ds378-GUS, or for the selection of unlinked transposition events with DsE and DsG (Figure 3). One advantage of this gene is its use as a reporter in whole tomato plants: at most developmental stages, spraying of Micro-Tom plants with 300 µg/ml kanamycin on three successive days, as previously described (Weide et al., 1989), allows identification of kanamycin-sensitive plants without their destruction. In such plants, the young leaves next to the shoot tip become white shortly after spraying (Figure 4A). Eventually, these leaves die but the subsequently emerging leaves are green and the plant survives. The hygromycin resistance gene indicates the presence of Ds378-GUS (Figure 4B). The indole acetic hydrolase (*iaaH*) gene confers

sensitivity to NAM. Sensitive plants develop a callus-like tissue at the root base and die about three weeks after germination (Figure 4C). NAM sensitivity can be used as a negative selection marker to select against Bam35S-Ac, thus stabilizing new insertions, and/or to select against the donor site in DsE and DsG. The *ALS* gene confers low resistance to 100 ppb chlorosulfuron in plants carrying an unexcised *Ds* element, and confers resistance to 2 ppm chlorosulfuron in plants where *Ds* excised (Figure 4D). In fact, all the selection features previously described for *Arabidopsis* (Fedoroff and Smith, 1993; Sundaresan et al., 1995) are also applicable to Micro-Tom and thus can be used for a transposon tagging system. The strategy for generating unlinked and stabilized transposition of *Ds*, and the strategy for selection of excision and reinsertion, where linked transposition events are most often recovered, have been previously described and compared (Sundaresan, 1996).

Using Ds378-GUS and Bam35S-Ac constructs, a new feature of the excision/reinsertion system emanates from the possibility to identify and rescue kanamycin sensitive plants (Figure 4A). Following crosses between Ds378-GUS- and Bam35S-Ac-carrying parents, selection of F2 plants for hygromycin resistance and kanamycin sensitivity enables the selection of unlinked, stabilized transposition events. This feature renders the system developed by Fedoroff and Smith (1993) a dual system suitable for selection of both linked and unlinked transposition events in tomato using the following procedure. First, the selection of $Hygr^r$ and $Kana^s$ plants enables to identify unlinked, stable transposition events. For this group of plants, NAM selection is unnecessary and chlorosulfuron should not be used, as the T-DNA containing the empty donor site segregated away. Second, the selection for plants resistant to chlorosulfuron among the $Hygr^r$ and $Kana^r$ plants, enables to identify linked transposition events. This group of plants is enriched in such events, because of *Ac* natural tendency for closeby transposition, and because of the elimination of some of the unlinked transposition events described above ($Hygr^r$, $Kana^s$ and $Chlorosulfuron^s$ plants).

The activity of the *Ac/Ds* system introduced into Micro-Tom was confirmed in F1 plants of a cross between transgenic plants transformed with Ds378-GUS and Bam35S-Ac

by sequencing *Ds* excision footprints. These footprints, shown in Figure 3 below the Ds378-GUS construct, are typical of what is expected for *Ac/Ds*. Interestingly, out of four clones analyzed, three had the same preferred footprint (GC inversion) as generated by *Ac* in the *wx-m7* allele of maize or in *Arabidopsis* (C. Weil personal communication) and tobacco (Gorbunova and Levy, 1997; Shalev and Levy, 1997), suggesting that preferential footprints formation, as described by (Scott et al., 1996), is species independent. In addition, GUS staining patterns in F1 plants found in roots of DsG X Bam35S-Ac (Figure 4E), in leaves (not shown) or in young fruits of Ds378-GUS X Bam35S-Ac (Figure 4F) indicated reintegration of *Ds* in or near genes in the course of plant development. In the Ds378-GUS parent, which has the weak Ac promoter, a faint GUS activity was detected only in the immature seeds of young fruits (data not shown).

Finally, transposition was confirmed in Southern blot analysis (Figure 5) of chlorosulfuron and hygromycin resistant F2 plants, progenies of cross Ds378-GUS X Bam35S-Ac. For this analysis, DNA from the *Ds*-containing parent, Ds378-GUS, was digested with *Hind*III, which cuts at the junction between the 5' end of *Ds* and the 5' of the GUS gene and does not cut in the T-DNA towards the left border (Figure 3). The GUS probe, present within *Ds*, revealed a single 8 kb band for the *Ds* parent (lane a), indicating that a single T-DNA copy is inserted in the genome. No hybridization was obtained, as expected, with the transposase parent (lane b). F2 plants showed variable hybridization patterns (Figure 5, lanes d-l) indicating element excision and reinsertion at new locations. Analysis of F2 plants from a cross between Ds378-GUS and Bam35S-Ac indicated that out of 22 plants, tested for chlorosulfuron resistance, 11 were resistant to hygomycin, showing root development and vigorous growth, when cut and transferred to hygromycin-containing medium. This makes the percentage of loss of excised *Ac* similar to previously reported figures in maize (Dooner and Belachew, 1989; Greenblatt, 1984; McClintock, 1956), tobacco (Jones et al., 1990) and Arabidopsis (Altmann et al., 1992) i.e. approx. 50%.

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CLAIMS

1. A method for large-scale mutagenesis and gene knockout in crop plants comprising the following steps:

(a) providing a miniature cultivar of a crop plant, said miniature cultivar having the following characteristics: (i) it is of reduced size in comparison to to the commercial cultivar of the same species; (ii) it can be grown at a plant density of at least 10-fold higher than standard growth conditions used for a commercial cultivar of the same species and can still yield reproductive organs under these conditions; and (iii) it is able to cross with a commercial cultivar of the same species;

(b) treating said miniature cultivar with a mutation-inducing agent or generating mutations of said miniature cultivar by insertion of a mobile DNA sequence; and

(c) identifying mutants or knockout of specific genes in said miniature cultivar by screening of large mutant-containing plant populations.

2. The method according to claim 1 wherein said crop plant is a crop plant useful as food, for ornamental purposes or for the wood industry.

3. The method according to claim 2 wherein the crop plant is tomato, maize, fruit trees, potato, or flowers.

4. The method according to any one of claims 1-3 wherein the miniature cultivar of the crop plant is developed from natural or induced mutations, by genetic engineering or by treatment with plant growth factors of the crop of interest.

5. The method according to claims 1-4 wherein new mutants or knockouts obtained in the miniature cultivar are transferred to a commercial background by standard crosses with the crop and by segregating out the miniaturizing gene(s) or transgene(s).

6. The method according to claim 1 (b) wherein the mutation-inducing agent is a chemical mutagen selected from ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), methyl-N-nitrosourea (MNU), and bleomycins.

7. The method according to claim 1 (b) wherein the mutation-inducing agent is irradiation selected from UV, γ-irradiation, X-rays and fast neutrons.

8. The method according to claim 1 (b) wherein the mutation of said miniature cultivar is generated by a mobile DNA sequence selected from T-DNA and transposons from the host plant or from an heterologous origin.

9. The method according to claim 1 wherein the identification of mutants in step (c) is done visually or by assaying for specific traits by conventional plant breeding techniques.

10. The method according to claim 1 wherein gene knockout in step (c) is achieved by detection of an insertion of a transposon or of T-DNA in a specific gene carried out by methods involving PCR screening using a first primer corresponding to a sequence of the transposon or of the T-DNA and a second primer corresponding to a sequence of the gene to be knocked out (target gene).

11. A method according to claims 1-10 wherein the crop plant is tomato.

12. A method according to claim 11 wherein the miniature cultivar of tomato is the miniature-dwarf-determinate *Lycopersicon esculentum* cultivar, Micro-Tom.

13. A method for large-scale mutagenesis and gene knockout in tomato comprising the following steps:

(b) treating miniature-dwarf-determinate *Lycopersicon esculentum* cultivar, Micro-Tom.with a mutation-inducing agent or generating mutations of said miniature cultivar by insertion of a mobile DNA sequence; and

(d) identifying mutants or knockout of specific genes in said miniature tomato cultivar by screening of large mutant-containing tomato plant populations.

14. A method for generating a library of mutants in crop plants comprising:

(a) introducing T-DNA or a transposable element into a miniature cultivar of a crop plant, said miniature cultivar having the following characteristics: (i) it is of reduced size in comparison to to the commercial cultivar of the same species; (ii) it can be grown at a plant density of at least 10-fold higher than standard growth conditions used for a commercial cultivar of the same species and can still yield reproductive organs under these conditions; and (iii) it is able to cross with a commercial cultivar of the same species;

(b) generating a large population of mutant plants of the miniature cultivar wherein each individual plant of said population contain a different T-DNA or transposon insertion;

(c) extracting DNA from pools of the mutant plants of step (b);

(d) identifying a pool of mutant plants containing an insertion in the gene to be knocked out (the target gene) by screening the DNA of the T-DNA- or transposon-carrying plants by PCR using a first primer corresponding to a sequence of the T-DNA or of the transposon and a second primer corresponding to a sequence of the target gene, and amplifying the PCR product only in the positive pools;

(e) identifying individual mutant plants from within the positive pool using DNA extracted from individual plants from the positive pool by PCR using a first primer corresponding to a sequence of the T-DNA or of the transposon and a second primer corresponding to a sequence of the target gene.

15. A method according to claim 14 wherein the transposable element is selected from an autonomous transposon, a non-autonomous transposon and an autonomous/ non-autonomous transposon system.

16. A method for generating a mutant tomato gene library comprising:

(a) introducing T-DNA or a transposable element into a miniature-dwarf-determinate *Lycopersicon esculentum* cultivar, Micro-Tom;

(b) generating a population of mutant plants of the Micro-Tom wherein each individual plant of said population contains a different T-DNA or transposon insertion;

(c) extracting DNA from pools of the mutant plants of step (b);

(d) identifying a pool of mutant plants containing an insertion in the gene to be knocked out (the target gene) by screening the DNA of the T-DNA- or transposon-carrying plants by PCR using a first primer corresponding to a sequence of the T-DNA or of the transposon and a second primer corresponding to a sequence of the target gene, and amplifying the PCR product only in the positive pools; and

(e) identifying individual mutant plants from within the positive pool using DNA extracted from individual plants from the positive pool by PCR using a first primer

corresponding to a sequence of the T-DNA or of the transposon and a second primer corresponding to a sequence of the target gene.

17. A method according to claim 16 wherein the transposable element is selected from an autonomous transposon, a non-autonomous transposon and an autonomous/ non-autonomous transposon system.

18. A method according to claim 17 wherein the transposable element system is the maize Ac/Ds transposon system.

For the Applicants

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Patent Attorney

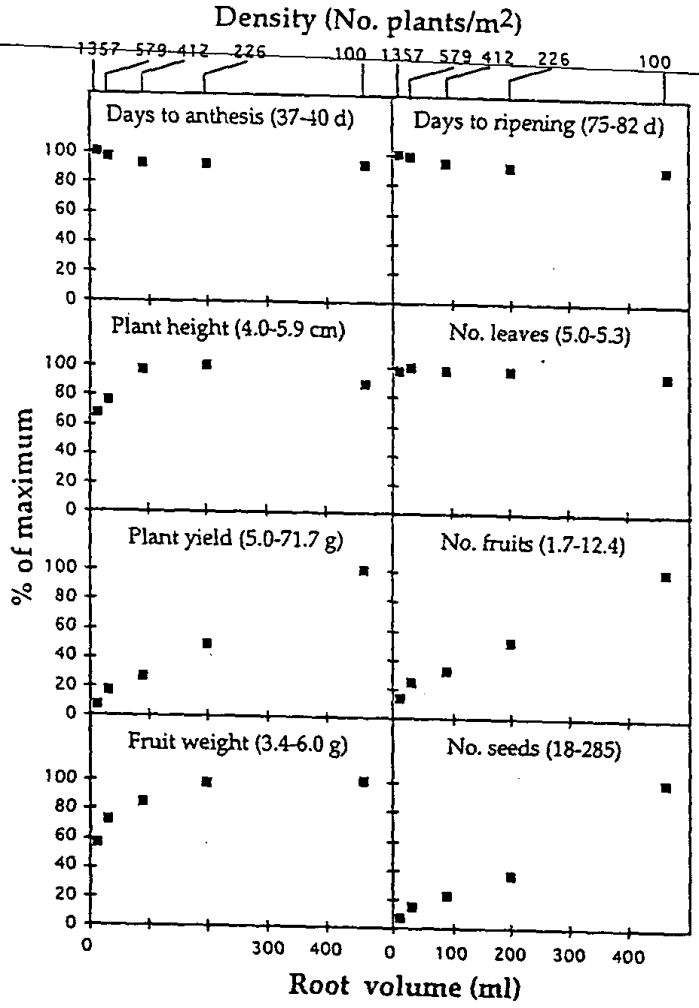

Density (No. plants/m2)
1357 579 412 226 100 1357 579 412 226 100
Days to anthesis (37-40 d)
Days to ripening (75-82 d)
Plant height (4.0-5.9 cm)
No. leaves (5.0-5.3)
Plant yield (5.0-71.7 g)
No. fruits (1.7-12.4)
Fruit weight (3.4-6.0 g)
No. seeds (18-285)
% of maximum
0 20 40 60 80 100
0 300 400 0 100 200 300 400
Root volume (ml)

Fig. 1

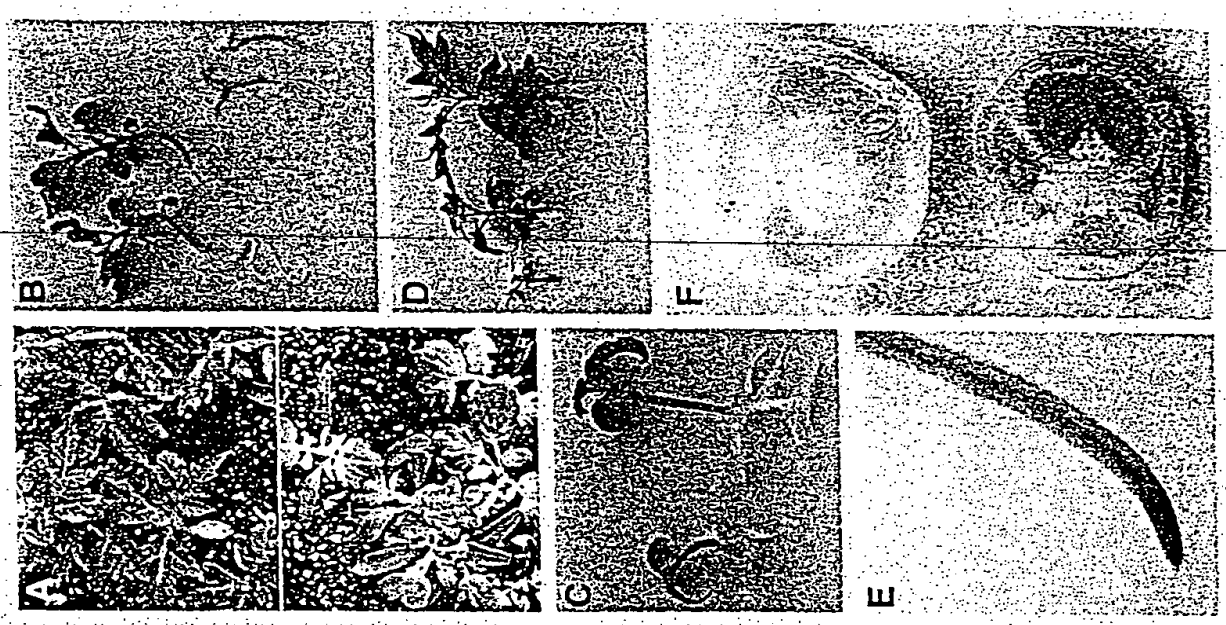


Fig. 4

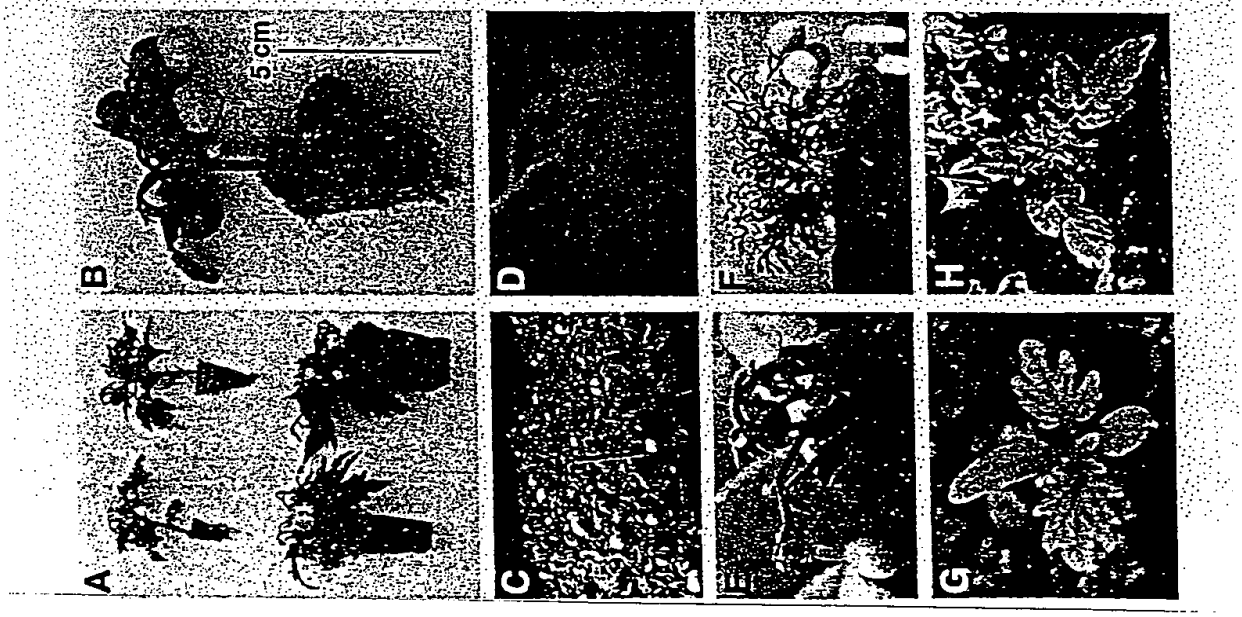
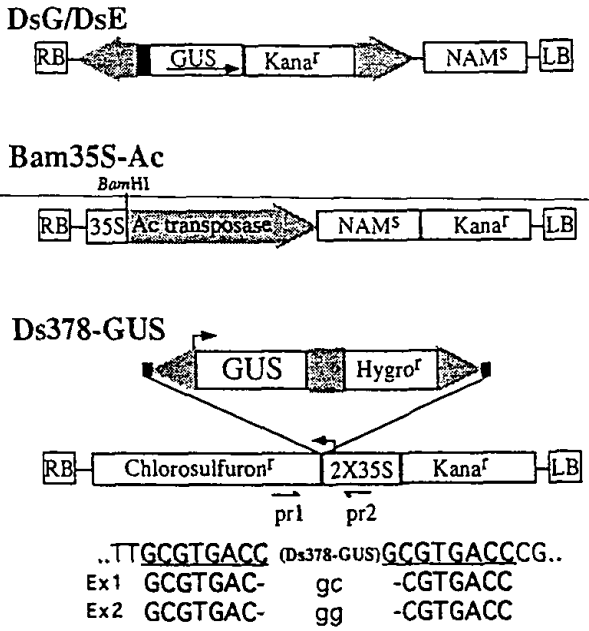


Fig. 2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NAM^s
LB
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BamH1
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LB
Ds378-GUS
GUS
Hygro^r
RB
Chlorosulfuron^r
2X35S
Kana^r
LB
pr1
pr2
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Ex2 GCGTGAC- gg -CGTGACC

Fig. 3

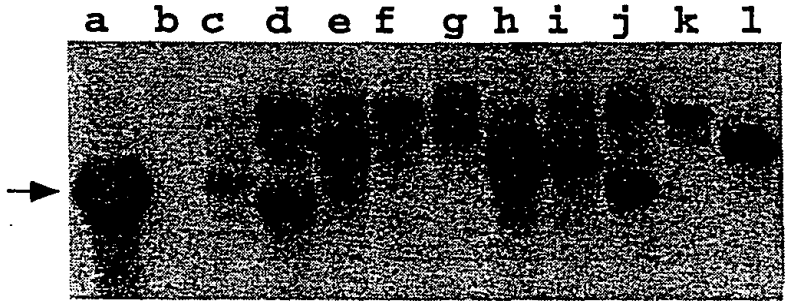
a b c d e f g h i j k l

Fig. 5